Loss of PDZK1 expression activates PI3K/AKT signaling via PTEN phosphorylation in gastric cancer

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Phosphorylation of PTEN plays an important role in carcinogenesis and progression of gastric cancer. However, the underlying mechanism of PTEN phosphorylation regulation remains largely elusive. In the present study, PDZK1 was identified as a novel binding protein of PTEN by association of PTEN through its carboxyl terminus and PDZ domains of PDZK1. By direct interaction with PTEN, PDZK1 inhibited the phosphorylation of PTEN at S380/T382/T383 cluster and further enhanced the capacity of PTEN to suppress PI3K/AKT activation. PDZK1 suppressed gastric cancer cell proliferation by diminishing PI3K/AKT activation via inhibition of PTEN phosphorylation *in vitro* and *in vivo*. The expression of PDZK1 was frequently downregulated in gastric cancer spe- cimens and correlated with progression and poor prognosis of gastric cancer patients. Downregulation of PDZK1 was associated with PTEN inactivation, AKT signaling and cell proliferation activation in clinical specimens. Thus, low levels of PDZK1 in gastric cancer specimens lead to increase proliferation of gastric cancer cells via phosphorylation of PTEN at the S380/T382/T383 cluster and constitutively activation of PI3K/AKT signaling, which results in poor prognosis of gastric cancer patients.

1. Introduction

Gastric cancer (GC), one of the most common fatal cancers, is the second leading cause of cancer related death worldwide [1,2]. Despite the improved prognosis of patients with GC resulting from early diagnosis, surgery and adjuvant therapy, 5-year survival rate remains low (28%–31%) owing to most patients diagnosed at an advanced stage accompanied by metastasis that limits therapeutic strategies [3,4]. The outcome for patients with metastatic gastric cancer is very poor, with median survival being ~12 months [1,2].

Gastric carcinogenesis was reported to be associated with several factors including geographical location, diet, *H. Pylori* infection, low gastric acidity and genetic factors. Therefore, GC is considered as a highly heterogeneous disease. During recent years, multiple molecular abnormalities underlying GC carcinogenesis and progression have been identified including p53, EGFR, TGF-β, PTEN, and PI3K/AKT et al. [5–9]. PI3K/AKT pathway is activated by diverse cellular stimuli regulating various physiological functions such as cell growth, cell survival, cell cycle progression, protein translation and metabolism. Mal- function of PTEN (loss of PTEN expression, gene mutation, or gene deficiency) plays a causal role in the aberrant activation of the PI3K/ AKT pathway. Studies reveal that loss of PTEN function underlies AKT activation is closely correlated with the initiation and development of activation is closely correlated with the initiation and development of gastric carcinoma [8,9]. PTEN deficiency due to mutation or deletion only contributes to a small fraction of PTEN dysfunction in gastric cancer [8,10,11]. PTEN function may be lost or regulated by many nongenomic mechanisms such as post-translational modification. Phosphorylation is one of the most common post-translational modifications for PTEN to regulate its function [12,13]. PTEN protein contains an N terminal phosphatase domain, a lipid-binding C2 domain, and a Cterminal tail. Phosphorylation of the PTEN C-terminal tail region, including phosphorylation of Ser380, Thr382, and Thr383, can reduce PTEN phosphatase activity [14–16], and significantly decrease its capacity to suppress carcinogenesis and progression [17,18]. PTEN contains class I PDZ binding motif (PBM) at its carboxyl terminus (-ITKV), which can associate with diverse PDZ proteins to regulate PTEN compartmentalization, cellular signaling [19,20], and phosphorylation et al. [16,21]. The outcomes of PTEN binding to different PDZ proteins are distinct. Interaction with PDZ protein MAGIs regulates the stability of PTEN and targets it to specialized compartments such as tight junctions in epithelial cells [22]. Interaction with MASTs kinases enhances PTEN phosphorylation [22,23]. Phosphorylation of threonine 382 and 383 decreases PTEN affinity with MAGI2 [24] and reduces PTEN stability [23], which subsequently diminishes its capacity to suppress PI3K/AKT signaling [25]. PTEN inactivation through phosphorylation can activate PI3K/AKT pathway and promote malignant cell growth and proliferation. Yang Z et al. reported that high-levels of PTEN phosphorylation (S380, or S380/T382/T383 cluster) were found in GC clinical specimens as compared with normal gastric tissues and the ratio of phosphorylated PTEN to total PTEN was higher in human GC cell lines than normal gastric epithelial cells [17,18]. These findings implicate a novel mechanism of loss function of PTEN in GC. Detailed regulatory mechanisms to modulate PTEN phosphorylation have remained largely illusive. Further understanding how PTEN activity is regulated may help to reveal the pathophysiological mechanisms of GC. Therefore, our study has been focused on finding potential factors to regulate phosphorylation and activity of PTEN upon binding with its carboxyl terminus. GST fusion proteins containing the last thirty amino acid residues of PTEN (PTEN-CT, encoding amino acid from 374 to 403) was purified and performed in pull down assay to screen PTEN-binding proteins. PDZK1 (PDZ Domain Containing1) was identified as a novel binding protein associated with PTEN-CT. PDZK1, also called CAP70 or NHERF3, belongs to NHERF family, which scaffolds a variety of proteins to modulate multiply signaling transduction by forming complexes [26].

In this study, we demonstrated that PDZK1 interacted with PTEN-CT and inhibited the phosphorylation of PTEN at S380/T382/T383 cluster, subsequently diminished PI3K/AKT signaling pathway. Loss of PDZK1 expression, which is frequently detected in gastric cancer specimens, promoted gastric cancer cell proliferation by enhancement of PI3K/AKT activation via upregulation of PTEN phosphorylation in vitro and in vivo studies. Therefore, our study presents evidences that loss of PDZK1 expression contributes to the carcinogenesis and progression of gastric cancer.

2. Materials and methods

2.1. Cell culture

U373 cell line was obtained from European Collection for Animal Cell Culture (ECACC, Porton Down, Salisbury, UK). COS7, 293 and the human gastric carcinoma cell lines AGS, BGC-823

and MGC-803 cells were obtained from the Cell Resource Center, Peking Union Medical College. Cells were grown in DMEM medium (COS7, 293, U373) or RPMI-1640 medium (BGC-823, AGS, MGC-803) supplemented with 10% fetal bovine serum (FBS) (complete medium) and 1% penicillin/ streptomycin under a humidified atmosphere of 5% CO 2 at 37 °C. All cell culture reagents were purchased from HyClone (Logan, UT).

2.2. Plasmid constructions, RNA interference (RNAi) and transfection

Constructs of myc-PDZK1 was kindly provided by Dr. Randy Hall (Emory University, Atlanta, GA), Flag-PTEN, GST-PTEN-wt and its variety point mutants of GST-PTEN-CT (ITKV/ITKA/ITAV/IAKV/ ATKV) were constructed as previously report [27]. For knockdown experiments, small interfering RNAs (siRNA) against PDZK1 were synthesized by Sigma (St Louis, MO). siRNAs and their sequences were as follows: siPDZK1#1, sense 5′-CAAAGAAACUGACAAGCGUdTdT-3′ and anti-sense 5′-ACGCUUGUCAGUUUCUUUGdTdT-3′; siPDZK1#2, sense 5′-GUCAAAUCAUCAAGGACAUdTdT-3′ and anti-sense 5′-AUGUCCUU GAUGAUUUGACdTdT-3′. The control siRNA (sc-37007) was obtained from Santa Cruz Biotechnology. The plasmid for stably overexpression (pcDNA-PDZK1) or knockdown PDZK1 (shPDZK1#1: 5′-TTTCAACCAC TTCCTCAT GGC-3′ and shPDZK1#2: 5′-TTTGACCTTTCTGTTCTG AGC-3′) were a kind gift from Dr. Michael R. Beard [28]. GV248shPTEN (short hairpin RNAs) (Ji Kai Gene, Shanghai, China) was used to knock down PTEN and the effects were verified by Western blot.

For transfection, cells were seeded into a 6-well plate and then transfected with 1 μg plasmids or 80 nmol/L siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For siRNA knockdown and transient transfection experiments, cells were collected 48 h post-transfection for protein extraction. For establishment of stable PDZK1-expressing cells, BGC-823 cells were transfected with control vector or constructs of PDZK1. For cells stably knockdown PDZK1, BGC823 cells were transfected with shControl or shPDZK1 plasmids. After 24 h transfection, the cells were selected with 800 μg/mL G418 (Calbiochem, San Diego, CA) for PDZK1 overexpressed cells, or using 300 ng/mL puromycin (Sigma, St Louis, MO) for cells transfected with shPDZK1 in culture medium for an additional 14 days. Expression of the PDZK1 protein was verified by Western blotting.

2.3. MALDI–TOF/TOF-MS analysis and database searching

Protein bands of interest were identified by combining peptide mass fingerprints and sequence tags obtained by MALDI-TOF/TOF-MS analysis, and Coomassie blue-stained spots of interested were cut into small pieces followed by washing, dehydration, and digestion as previously described [29]. The mixture was sonicated for 10 min and centrifuged. The supernatant was subjected to matrix-assisted laser-desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) by using the prOTOF 2000 MALDI O-TOF mass spectrometer (PerkinElmer/ Sciex, Fremont, CA) operated in the delayed-extraction and linear mode. External calibration for all mass spectra by using a standard peptide mixture (angiotensin II and ACTH 18–39) was performed. Acquisition of mass data was piloted by TOF Works software (Perkin Elmer/Sciex). The mass spectra data were matched with the NCBInr database (http://www.matrixscience.com) for the identification of proteins with the following parameters: monoisotopic; peptide mass tolerance, ± 100 parts per million; fragment mass tolerance, ± 0.5Da; and missed cleavages, 1.

2.4. GST pull-down assay

Purification of GST- or His-tagged proteins and GST pull-down assay were performed as described previously [30]. Briefly, equal amount of purified GST fusion proteins (conjugated on beads) were incubated with 1 ml cell lysates containing His-tagged PDZ domains of PDZK1 proteins. Bound proteins were then eluted by boiling in 2 × sample buffer, separated by SDS-PAGE, and analyzed by Western blotting.

2.5. Co-immunoprecipitation and Western blotting

Lysis of cells, co-immunoprecipitation and Western blotting were performed as described previously [31]. The immunoprecipitated proteins were separated by SDS-PAGE and subjected to Western blot analysis. A small fraction of cell lysates was also separated by electrophoresis on the SDS-PAGE to examine the expression of proteins in whole cell lysates.

2.6. Over-lay assay

Equal amounts of His-PDZK1 PDZ fusion proteins (1 μg) were run on 12% SDS–PAGE gels and transferred into nylon membranes. The membranes were blocked and then overlaid with GST-PTEN-CT at different concentrations. The blots were then washed three times with wash buffer and incubated with anti-GST-HRP antibody (1:5000, MBL), and then visualized via chemiluminescence using the ECL kit (Applygen, Beijing, China).

2.7. Cell proliferation assay

Cells were cultured in 96-well microplates. At 0 h, 24 h, 48 h, 72 h and 96 h after plating, CCK-8 (Dojindo, Kumamoto, Japan) was added to each well according to the manufacturer's instructions and the cells were cultured for another 1 h. Viable cells were quantified by measuring absorbance at 450 nm with an EnSpire label microplate reader (PerkinElmer, Waltham, MA).

2.8. Colony-formation assay

Cells were digested in 0.25% trypsin to reconstitute the single-cell suspension at a density of 1.0 × 10 5 cells per ml. Cell suspensions were plated onto 6-well plates and incubated at 37 °C and at an atmosphere of 5% CO 2 for 14 days. The supernatants were discarded, and the colonies washed with PBS for twice and fixed in methanol for 10 min. Cells were then stained with crystal violet and allowed to air dry at room temperature. The experiments were triplicated and the numbers of colonies containing more than 50 cells were microscopically counted to calculate the colony formation rate as number of colonies/number of cells ×100% divided by control.

2.9. Tumor growth assay in animal models

All animal experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Care Committee of Capital Medical University. Control or stable PDZK1-expressing BGC-823 cells (1 × 10 5) in 0.1 mL PBS were subcutaneously injected into the dorsal flank of male Balb/c nude mice (age of 4–5 weeks). Each group included 10 mice. The mice were monitored every day for the growth of tumors. The tumor volume was calculated with the following formula: $V=(\pi/6)x$ length x width 2 . The tumor xenografts were dissected and weighted after the sacrifice of the mice.

2.10. Datasets collection

A total of 158 clinical GC specimens including 36 paired tumor and adjacent non-tumor samples, which were collected from Beijing Friendship Hospital, Capital Medical University, China from 2008 to 2012, were used in this study. The specimens were obtained during surgical resection or endoscopy at the Friendship Hospital. None of these patients received preoperative chemotherapy or radiotherapy. Informed consents were obtained from all patients and the study protocol was approved by the Clinical Research Ethics Committee of the Capital Medical University. The TCGA data for mRNA expression and the corresponding clinical data from Synapse (http://synapse.org; syn1461177) were downloaded and processed using standard methods. RNA-Seq analysis used data from 415 stomach cancers and 35 adjacent normal tissues.

2.11. Immunohistochemistry analysis

The xenograft tumors and GC samples tissues were fixed with 4% formalin and prepared as paraffin-embedded section. The tissue slides were deparaffinized in xylene and rehydrated through graded alcohol baths. Sections were either stained with hematoxylin and eosin (KeyGen Biotech) for morphological evaluation, or antigen retrieval for IHC studies. For antigen retrieval methods, slides were submerged into EDTA antigenic retrieval buffer and microwaved, followed by treatment with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity and incubation with 3% bovine serum albumin to block the nonspecific binding. Rabbit polyclonal anti-PDZK1 (1:100; Abcam), anti-PTEN (1:100; Cell Signaling) and anti-phospho-PTEN (S380/T382/T383) (1:100; Cell Signaling) antibody were incubated with the tissues overnight at 4 °C followed by incubation with horseradish-peroxidase (HRP) -conjugated secondary antibody kits. Afterwards, the expression of PDZK1 in tissue slides was assessed by two pathologists who were blinded to clinical and pathologic information. The intensity of immunostaining was scaled as no stain (1), weak staining (2), moderate staining (3), or strong staining (4). Negative control reactions were obtained by omitting primary antibodies. Image-Pro Plus software was also used to analyze optical density of PDZK1, PTEN, and pPTEN expression.

2.12. Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed as previously [31]. To assess whether genes from pre-defined gene set of PTEN signaling inactivation (PTEN_DN.V2_UP), cell apoptosis (GO_EXECUTION_PHASE_OF_APOPTOSIS), AKT activation (AKT_UP.V1_UP) and cell proliferation (CELL_PROLIFERATION_GO_0008283) obtained from http://www.broadinstitute.org were enriched among the highest- (or lowest-) ranked genes or distributed randomly by calculating a pathway Enrichment Score (ES). Default settings were used and thresholds for significance were determined by permutation analysis (1000 permutations). False Discovery Rate (FDR) was calculated. Significant enrichment was considered when the FDR score was less than 0.25.

2.13. Antibodies and reagents

The anti-His antibody was purchased from MBL (Nagoya, Japan). The anti-myc antibody was purchased from Applygen (Beijing, China). Anti-PDZK1 was purchased from Abcam (Cambridge, UK), anti-phosphor-PTEN (S380/T382/T383), anti-PTEN, anti-phosphor-AKT and anti-AKT were purchased from Cell Signaling (Danvers, MA). AntiFLAG M2 antibody and FLAG M2-agarose were purchased from Sigma (St Louis, MO). Antibodies of anti-GAPDH, anti Ki67 and HRP-conjugated secondary antibodies were obtained from ZSGB-BIO (Beijing, China).

2.14. Statistical analysis

The difference between tumor and para-cancerous tissues in PDZK1 protein or mRNA expression was analyzed by nonparametric Mann–Whitney test or unpaired t-test. Independent samples t-test was performed to analyze the statistical difference between two groups of cells in colony formation. Kaplan-Meier survival curve and log-rank test were used to evaluate overall survival data corresponding to PDZK1 expression. The differences in cell viability and nude mice tumor size between two groups were determined by repeated-measures analysis of variance. All statistical tests were performed using Graphpad Prism 5.0 (Graphpad Software, Inc., San Diego, CA) or the SPSS program (version 17.0; SPSS, Chicago, IL). Data were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. PDZK1 binds to PTEN via PTEN-CT and PDZ domains of PDZK1

In order to find potential PTEN binding proteins, rabbit stomach tissue lysates were used to screen PTEN-binding proteins by GST pulldown experiments. As the results shown, GST protein bound nonspecifically to multiple bands, whereas GST-PTEN-CT was associated with several

extra bands (Fig. 1A). Interestingly, among these binding proteins, a band at about 70 kD (*) was detected with a very strong signal. The band was further analyzed by MS spectrometry and identified as PDZK1 (Fig. 1B). Another band at ~50 kD (#) was recognized as NHERF1, which is consistent with finding from our previous report

[27]. To verify the interaction of PTEN with PDZK1 in vitro, GST-PTENCT was used for pull-down assays from lysates of normal stomach tissues, and pull-down complexes were probed with an anti-PDZK1 antibody, with the specificity of antibody was verified in our previous study

[31]. As shown in Fig. 1C, there was a robust PDZK1 signal in the GSTPTEN-CT pull-down complex, but no signal was detected in GST alone pull-down complex, indicating that GST-PTEN-CT associated selectively with PDZK1. To examine the interaction of endogenous PTEN and PDZK1, solubilized stomach tissue lysates were incubated with antiPDZK1 antibody linked to protein A/G-agarose beads. A robust PTEN signal was detected in PDZK1 co-immunoprecipitation complexes, but no signal was detected in the control samples (Fig. 1D), which indicated the existence of a physical complex between PTEN and PDZK1 in native tissues.

PDZK1 possesses four PDZ domains, and the PBM of the PTEN-CT belongs to the canonical class I PDZ domain-binding motif. To clarify the structural determinants of the PTEN/PDZK1 interaction, individual His-PDZ domains of PDZK1, PTEN-CT wild type (PTEN-CT-ITKV) and a series of single point mutations of PTEN-CT with Ala substitution in the ITKV motif of wild type PTEN-CT (PTEN-CT-ITKA, ITAV, IAKV, ATKV) were constructed (Fig. 2A). In our GST pull down studies, strong signals of PDZ2 and PDZ3 domains of PDZK1 were detected in the GST-PTENCT pull-down complex, and conversely only a very weak signal for HisPDZK1-PDZ1 domain and no signal for His-PDZK1-PDZ4 domain was detected (Fig. 2B), suggesting that PDZ2 and PDZ3 domains were the principal domains mediating the interaction. To determine the binding affinity of PTEN-CT with PDZK1, overlay assay was performed with different concentrations of PTEN-CT proteins. The estimated binding affinity of PDZ2 and PDZ3 with PTEN-CT was about 20 nM and 25 nM respectively (Fig. 2C). To examine whether PTEN-CT could associate with PDZK1 via the PBM of PTEN, purified fusion proteins of wild type GST-PTEN-CT-ITKV or its point-mutated versions were used to pull down the lysates of COS7 cells expressed Myc-PDZK1. Pull down precipitates were then subjected to Western blot with anti-myc antibody (Fig. 2D). Results showed that PTEN-CT-ITKV was strongly associated with Myc-PDZK1, whereas the mutation of any one of the terminal four amino acid residues to Ala almost completely abolished this interaction. To further investigate the binding specificity between PDZ domains of PDZK1 and PTEN-CT, GST-PTEN-CT-ITKV or GST-PTEN-CT-ITKA was used to pull down His-PDZ2 or His-PDZ3 of PDZK1. His-PDZ2 and HisPDZ3 were only identified in wild type GST-PTEN-CT pull down complexes. These data indicated that when the last amino acid of PTEN-CT- ITKV was mutated, the interaction of PDZ domains of PDZK1 with PTEN-CT was completely abolished (Fig. 2E). These findings demonstrated that PDZK1/PTEN interaction was mediated by the PDZ domains of PDZK1 and PBM of PTEN. To further verify these results in cells, co-immunoprecipitation experiment was performed using antiFlag antibody to immunoprecipitate Flag-tagged full length of wild type PTEN and its mutant PTEN-ITKA respectively. A robust PDZK1 signal was detected in wild type PTEN, but not Flag-PTEN-ITKA co-immunoprecipitation complexes (Fig. 2F), which demonstrated the interaction between PTEN and PDZK1 is specifically mediated by the Cterminal of PTEN.

3.2. PDZK1 inhibits PTEN phosphorylation and suppresses the activation of PI3K/AKT signaling in gastric cancer cells

It has been reported that PTEN was phosphorylated in its C-terminal tail by several kinases [23]. In order to delineate the function of PDZK1/PTEN interaction, the roles of PDZK1 in the regulation of PTEN phosphorylation was investigated in gastric cancer cells. Firstly, PDZK1 was overexpressed in BGC-823 gastric cancer cells and the levels of phosphorylated PTEN were analyzed. As shown in Fig. 3A, PTEN phosphorylation at S380/T382/T383 was inhibited in BGC-823 cells when PDZK1 was overexpressed, whereas total PTEN protein levels remained

unchanged. Moreover, PDZK1 was detected to inhibit PTEN phosphorylation in a dose dependent manner in COS7 cells, an ideal cell line generally used in cell signaling study because of its high efficiency of transfection and feasibility of maintenance (Fig. S1A). To verify this result, BGC-PDZK1 cells, the BGC-823 gastric cancer cells which stably overexpressed PDZK1, were transiently transfected with siPDZK1, and the phosphorylation of PTEN was detected by Western blotting. Results showed that PDZK1 expression was successfully knocked down by siPDZK1 in BGC-PDZK1 cells, and the levels of phospho-PTEN (S380/T382/T383) were recovered (Fig. 3B). Consistent results were also obtained from another gastric cancer cell line, MGC803 cells (Fig. S1B). To investigate whether the inhibition of PTEN phosphorylation by PDZK1 was PTEN/PDZK1 interaction-dependent, the constructs of Flag tagged full length of wild type PTEN (Flag-PTENITKV) or its point mutant Flag-PTEN-ITKA was co-transfected with mycPDZK1 into COS7 cells, and the PTEN phosphorylation was analyzed by Western blotting. As shown in Fig. 3C, PDZK1 reduced the levels of phospho-PTEN (S380/T382/T383) in cells transfected with wild type PTEN. However, ectopic expression of PDZK1 did not reduce the levels of phospho-PTEN (S380/T382/T383) in cells transfected with PTENITKA, in which the association was interrupted (Fig. 2F). The levels of phosphorylated wild type PTEN were lower than that of PTEN-ITKA when these constructs were transfected with the same dose. These data support the notion that endogenous PDZK1 in COS7 cells can regulate PTEN phosphorylation and this effect is magnified by the overexpression of exogenous PDZK1. These results demonstrated that the PDZK1/PTEN interaction was required for PDZK1 suppression of phospho-PTEN (S380/T382/T383).

PTEN is a major negative regulator of the PI3K/AKT signaling pathway. To confirm PDZK1 inhibition of PTEN activation in gastric cancer cells, AKT phosphorylation was further investigated. As shown in Fig. 3D, the levels of phospho-PTEN (S380/T382/T383) were coordinately decreased in BGC-823 cells as the expression of PDZK1 was increased. The phosphorylated AKT (pAKT) was suppressed by PDZK1 in a dose dependent manner, which is consistent with the observations from AGS gastric cancer cells (Fig. S1C). Furthermore, the levels of phosphorylated AKT were increased when PDZK1 expression was inhibited by siRNA in BGC-823 cells (Fig. 3E). These results provide further evidences for an inhibitory role of PDZK1 in PI3K/AKT activation. Moreover, PDZK1 failed to block AKT phosphorylation in BGC-shPTEN cells (Fig. 3F), indicating that inhibition of AKT activation by PDZK1 was PTEN-dependent. These findings provide evidences that PDZK1 inhibits activation of AKT signaling in a PTEN-dependent manner in GC cells. Similar results were obtained from U373 glioblastoma cells, in which PTEN was deficient. Overexpression of PDZK1 did not alter the levels of pAKT in U373 cells. However, when U373-PTEN cells, in which PTEN was stably expressed in U373 cells, were transiently transfected with myc-PDZK1, pAKT levels were reduced (Fig. S1D). These findings further confirmed that PTEN is essential for PDZK1 inhibition of AKT signaling activation.

3.3. PDZK1 inhibits the proliferation of gastric cancer cell

PTEN regulates a number of cellular processes such as proliferation mainly through the PI3K/AKT signaling pathway [32,33]. To investigate the effects of PDZK1 in gastric cancer cells proliferation, PDZK1 was either stably expressed or knocked down in BGC-823 and AGS cells respectively. The proliferation rates of BGC-PDZK1 (Fig. 4A) and AGS-PDZK1 (Fig. 4B) cells were slower than that of control cells as determined by CCK-8 assay. Knockdown of PDZK1 expression by stable transfection of shRNA#1or shRNA#2 promoted proliferation of BGC823 (Fig. 4C) or AGS cells (Fig. 4D). To further evaluate the effects of PDZK1 on GC cell growth and proliferation, colony formation assay was performed. Data showed that the colony formation rate was reduced up to 40%–45% as compared with the controls after overexpression of PDZK1 in BGC-823 (Fig. 4E, top panel) and AGS cells respectively (Fig. 4E, bottom panel). Consistently, the number of colony formation in BGC-823 (Fig. 4F, top panel) or AGS (Fig. 4F, bottom panel) cells, in which PDZK1 was stably knocked down, was significantly larger than that of control cells. These data indicated that PDZK1 could inhibit the proliferation of gastric cancer cells.

To test whether PDZK1 inhibition of cell proliferation was mediated by PTEN, CCK-8 assay was performed with or without PDZK1 overexpression to analyze the proliferation of BGC-shPTEN

cells, in which PTEN was stably knocked down by shRNA. As shown in Fig. 4G, overexpression of PDZK1 inhibited proliferation of BGC-823 gastric cancer cells, which was consistent with the results of Fig. 4A. Stably knockdown of PTEN promoted proliferation of BGC-823 cells as compared with the control cells, which was in agreement with the roles of PTEN as a tumor suppressor. However, when PTEN expression was knocked down, the proliferation of BGC-823 cells was not different in the presence or absence of PDZK1 ($P > 0.05$). This result was further verified in PTEN-deficient U373 cells. Overexpression of PDZK1 did not affect the proliferation of U373 cells (Fig. S2A). However, PDZK1 could significantly inhibit the proliferation of U373 cells when exogenous PTEN was expressed (Fig. S2B). These results indicated that PDZK1 inhibition of gastric cancer cells proliferation was dependent on PTEN expression.

3.4. PDZK1 inhibits growth of human gastric-carcinoma xenograft in nude mice and suppresses PTEN phosphorylation in xenograft tumors

To examine the biologic effects of PDZK1 expression in vivo, a xenograft tumor model was established by subcutaneous injection of control or BGC-PDZK1 cells into nude mice. As shown in Fig. 5, overexpression of PDZK1 significantly inhibited BGC-823 cell proliferation in vivo. Accordingly, the weight of tumors (Fig. 5A, $P < 0.05$) and the tumor volume (Fig. 5B, $P < 0.05$) in the group implanted with BGCPDZK1 cells were significantly lower than those of the control. These findings suggested that PDZK1 delayed tumor growth. To further verify whether PDZK1 suppresses PTEN phosphorylation in xenograft tumors, the expression of PDZK1 and phospho-PTEN (S380/T382/T383) were examined in xenograft tumors by both Western blotting and immunohistochemistry (IHC) staining respectively. Results showed that robust expression of PDZK1 significantly reduced the levels of phosphor- PTEN (S380/T382/T383) and similar total levels of PTEN were detected in BGC-PDZK1 xenograft tumors (Fig. 5C). The effects of PDZK1 in cell proliferation and apoptosis were further verified in xenograft tumors. It showed relatively weak intensity of phosphorylated AKT and Ki67 staining and greater number of TUNEL positive cells in BGCPDZK1 xenograft tumors when compared with the control (Fig. 5D). Taken together, these results indicated that by blocking PTEN phosphorylation, PDZK1 increased apoptosis, suppressed cell proliferation, and resulted in inhibition of BGC-823 gastric-carcinoma xenograft growth in nude mice.

3.5. PDZK1 is negatively correlated with tumor grade and prognosis of GC

To determine the clinical relevance of PDZK1 expression in gastric cancer, clinical specimens from Chinese GC patients ($n = 158$) were analyzed. As shown in Fig. 6A, the protein levels of PDZK1 were significantly downregulated in GC patients as compared with its adjacent tissues ($P < 0.05$). The representative images of immunohistological staining of PDZK1 in cancer specimens were shown in Fig. 6B. Down regulation of PDZK1 was also verified in mRNA levels in the TCGA cohort of GC patients ($n = 450$) (Fig. 6C, $P < 0.05$). In order to understand whether expression of PDZK1 correlates with tumor development, the mRNA levels of PDZK1 in different stages and grades of GC patients were analyzed. Data showed that as compared with GC patients in T1/T2 stages, the levels of PDZK1 mRNA were much lower in T3/T4 stages in TCGA GC patients (Fig. 6D, $P < 0.01$). The expression level of PDZK1 was negatively correlated with the tumor grade either at protein (Fig. 6E, $P < 0.01$) or mRNA (Fig. 6F, $P < 0.01$) levels, indicating that low levels of PDZK1 were correlated with tumor development. To evaluate the correlation of PDZK1 levels with the prognosis of GC patients, clinical specimens from Chinese GC patients with overall survival information were then divided into high and low PDZK1 expression groups, and Kaplan-Meier survival curve was analyzed. Results showed that patients with high PDZK1 levels had better outcomes than those with low PDZK1 expression (Fig. 6G, $P < 0.05$). Similar results were also obtained from TCGA database clinical GC specimens (Fig. 6H, $P < 0.05$). These findings indicated that PDZK1 expression levels were significantly negatively correlated with the prognosis of GC patients. To determine the association of PDZK1 with PTEN expression in the tumorigenesis and progression of GC, TCGA data set of GC patients with PTEN LOH were obtained. There was no statistic difference for the overall survival of GC patients with LOH of PTEN regardless of PDZK1 levels (Fig. S3, $P > 0.05$), which indicated that PDZK1 suppression of gastric cancer patient progression was mediated by PTEN.

To further explore the correlation of PDZK1 and phospho-PTEN levels in gastric cancer, serial sections of gastric cancer and adjacent normal tissue were stained for PDZK1, PTEN and phospho-PTEN (S380/ T382/T383) by IHC respectively. Similar results were observed in PDZK1, PTEN and phospho-PTEN (S380/T382/T383) staining sections as compared with results of xenograft tumor studies (Fig. 5D). PTENpositive cells were widely distributed in either GC or adjacent tissues.

PDZK1 immunoreactivity was downregulated concomitantly with upregulation of phospho-PTEN (S380/T382/T383) in GC cells as compared with adjacent normal cells (Fig. 7A). In order to understand whether PTEN activity is correlated with GC development and prognosis in clinical specimens, gene signatures in response to PTEN inactivation were analyzed and data showed that PTEN inactivation was enriched in clinical GC specimens with poor prognosis (< 5 years survival) (Fig. 7B), suggesting that PTEN inactivation was positively associated with poor prognosis of GC patients. To further verify whether PTEN signaling inactivation could be attributed to low level of PDZK1, GSEA was performed to test the enrichment value of PTEN inactivation associated with PDZK1 mRNA levels. Results from GSEA for TCGA database of GC patients showed that gene signatures of PTEN inactivation were robustly enriched in PDZK1 low levels group (FDR = 0.06), indicating that PTEN activity was positively correlated with PDZK1 levels (Fig. 7C). Since PTEN can inhibit PI3K/AKT signaling to suppress cell proliferation, we furthered analyzed the correlation of gene signatures of AKT signaling, cell proliferation and apoptosis with PDZK1 levels. Results showed that gene signatures of AKT signaling (Fig. 7D) and cell proliferation (Fig. 7E) were significantly enriched in group with low PDZK1 levels, and gene signatures of cell apoptosis were significantly enriched in group with high PDZK1 levels (Fig. 7F). These data indicated that low levels of PDZK1 in GC were positively correlated with PTEN inactivation, reduced apoptosis, and increased cellular proliferation. These findings provided first lines of evidences that PDZK1 deficiency in GC patients may contributes to the development and short survival of GC via inactivation of PTEN.

4. Discussion

Complex molecular mechanisms and signaling pathways are implicated in the carcinogenesis and progression of GC. There are evidences indicating that phospho-PTEN (S380/T382/T383) directly contributes to GC carcinogenesis and progression. However, the detailed molecular mechanisms about regulating PTEN phosphorylation remain unknown. Thereafter, investigating the molecular mechanisms that contribute to the phosphorylation of PTEN may provide clues in finding potential drug targets for cancer therapy, or potential biomarkers for GC diagnosis. In this study, we identified PDZK1 as a novel binding partner for PTEN and regulated PTEN/AKT signaling. Loss of PDZK1 expression promoted gastric cancer cell proliferation by enhancement of PI3K/AKT activation via upregulation of PTEN phosphorylation. Based on the finding from downregulation of PDZK1 in GC specimens at both mRNA and protein levels, our present study further suggests that loss of PDZK1 in GC is a novel mechanisms in associated with GC carcinogenesis via regulating PTEN phosphorylation.

Interaction of PTEN with PDZ protein have been considered as an important mechanism of tissue-specific regulation of PTEN functions [22,34]. Recently, MAGIs and NHERF1 have been reported to associate with PTEN to regulate PI3K/AKT signaling to inhibit tumor formation and progression, including cell growth, survival and migration [35–37]. However, none of these studies reported physiological and pathological significance of PTEN-PDZ interaction in gastric cancer cells. We analyzed TCGA database and found that MAGI-1 expression levels were unchanged in GC specimens (Fig. S4A). Although the mRNA levels of MAGI-2/3 were all downregulated (Figs. S4B–C), GSEA data indicated that MAGI-3 (Fig. S4F) failed to associate with gene signatures of PTEN inactivation in GC specimens. MAGI-2, however, was positively correlated with PTEN inactivation in GC specimen (Fig. S4E), which is controversial with the report that MAGI-2 enhanced PTEN stability [25], suggesting that MAGI-1/2/3 is not likely involved in PTEN inactivation in GC specimens. NHERF1 has been shown to contribute to the inactivation of AKT in tumors [38], but the NHERF1 protein was overexpressed in the majority of

gastric carcinoma tissues [39,40]. Thus, none of these reported PTEN binding PDZ proteins including NHERF1 and MAGI-1/2/3 were likely contributing to inactivation of PTEN/AKT signaling in GC specimens. In this study, we found that loss of PDZK1 expression in GC promoted gastric cancer cell proliferation by enhancement of PI3K/AKT activation via upregulation of PTEN phosphorylatio. Although NHERF1 and PDZK1 both belong to NHERF family, differential outcomes of NHERF1 and PDZK1 binding with PTEN suggest that selective binding preference of PTEN with specific PDZ proteins may distinctively affect the physiological significance of PTEN due to cell types and/or tissue specificity.

As a scaffold protein, PDZK1 likely plays dual roles in carcinogenesis depending on the tissue specificity. Ghosh et al. showed that PDZK1 was an oncogenic factor in breast cancer, and Kim et al. found that PDZK1 elicited an oncogenic role in breast cancer cell proliferation via increasing the stability of Akt [41,42], which is in line with our findings which show that upregulation of PDZK1 was detected and gene signatures of AKT activation was enriched in ER positive breast cancer patients with high levels of PDZK1 (Figs. S5A–C), indicating that PDZK1 promotes Akt activation and carcinogenesis in ER positive breast cancer. However, PDZK1 was identified as a novel tumor suppressor in ccRCC [31]. In this study, we found that PDZK1 suppressed PTEN phosphorylation, AKT signaling (Fig. 3) and GC cell proliferation (Figs. 4–5). Downregulation of PDZK1 in GC specimens and its correlation with tumor grade, stage and prognosis of GC patients (Figs. 6–7) suggests a tumor suppressive role of PDZK1 in GC. However, although PDZK1 elicits a tumor suppressive role in both ccRCC and GC, the molecular mechanisms within these two types of cancer are distinct. In kidney cancer tissues, PDZK1 blocked oncogenic signaling of SHP-1 via PLCβ3 [31]. However, no correlation of SHP-1 signaling with PDZK1 was detected in TCGA dataset of gastric cancer (Fig. S6). In addition, SHP-1 played a tumor suppressive role in GC [43–45] and low levels of SHP-1 were expressed in various gastric cancer cells [45], indicating that PDZK1 inhibition of gastric cancer cell proliferation was independent on targeting SHP-1 signaling. Thus, PDZK1 likely elicits tumor suppressive effects through a novel mechanism different from that reported in ccRCC.

PTEN is mainly present in the cytosol-membrane, where it dephosphorylates PIP3 to negatively regulate PI3K/AKT pathway, and further blocks cell proliferation and tumorigenesis [46]. The components of PI3K/AKT signaling pathway such as PI3K, PIP3 and AKT have been detected at both the cytoplasm and the nucleus [46–49]. In addition, increased levels of nuclear PTEN have been considered involved in regulating cell cycle progression [50]. In the present study, localization of phospho-PTEN (S380/T382/T383), PTEN and PDZK1 were detected in both cytosol and nucleus (Figs. 5D and 7A), which indicates that PTEN and PDZK1 interaction may occur and regulate PI3K/AKT pathway both in cytosol and nucleus. However, the biological significance of AKT activation in the nucleus largely remains unknown.

It has been reported that the PTEN C-terminus is phosphorylated by several protein kinase such as GSK3β, CK2, and MAST205 related kinases. Evidences showed that PTEN switches between open and closed states upon change of its phosphorylation status [15], and phosphorylation of PTEN favors the closed conformation, thereby retards its activation [15,51]. Phosphorylation of PTEN by MAST205 is indispensable to its PDZ domain, suggesting that PDZ domain recognition could be a specific mechanism that targets PTEN phosphorylation by these kinases [23,52,53]. In our unpublished data, PDZK1 competed with MAST205 to bind with PTEN. Since PDZK1 is a scaffold protein, it is possible that PDZK1 may sequester access of MAST205 to PTEN then leads to inhibition of PTEN phosphorylation by MAST205. The region of PTEN that is phosphorylated by GSK3β and CK2 (amino acids 360–386) is adjacent to the PDZ-binding motif [28,52], thus it is possible that PDZK1 could also obstruct the association of PTEN with CK2 or GSK3β, and retard PTEN phosphorylation. These hypotheses need further investigation. PDZK1 binding with PTEN may promote an open state of PTEN to maintain its phosphatase activity. Therefore, we propose a model in which low levels of PDZK1 in gastric cancer cells leads to reduction of PDZK1/PTEN binding, resulting in phosphorylation of PTEN C-terminal region then modulate the intramolecular interaction between the N-terminal region of the catalytic domain and C2 domains of PTEN to keep PTEN in an inactive closed conformation. Loss of

PTEN activity then leads to increased AKT activation and gastric cancer cell proliferation (Fig. 7E).

In summary, our study identified PDZK1 as a novel binding partner with PTEN. By direct interaction with PTEN, PDZK1 inhibits the phosphorylation of PTEN at S380/T382/T383 cluster and further enhances the capacity of PTEN to suppress PI3K/AKT pathway activation, which further results in inhibition of growth and proliferation of gastric cancer cells both in vitro and in vivo. We also verified the association of PDZK1 expression levels with phospho-PTEN (S380/T382/T383), the activation of PTEN signaling pathway, and cell proliferation within GC specimens. These findings indicate that PDZK1 deficiency contributes the GC carcinogenesis or progression via constitutively activated PTENPI3K/AKT signaling. Therefore, loss of PDZK1 expression contributes to the development and progression of GC, and low levels of PDZK1 serve as a potential prognosis biomarker for GC patients.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

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Legend to figures

Fig. 1. PDZK1 was identified as a novel binding protein of PTEN. (A) Screening of PTEN-CT binding partners by GST pull-down assay. Purified GST-PTEN-CT fusion proteins were adsorbed to glutathion-agarose beads, and used to pull down lysates of rabbit stomach tissues. The precipitates were run on SDS-PAGE gel, and then visualized by Coomassie blue staining. Positions of molecular weight markers are shown on the left. Coomassie blue staining revealed equal loading of the fusion proteins (bottom panel). (B) PDZK1 was detected as a novel PTEN associated protein by mass spectrometry. The protein band*, which strongly bound to the PTEN-CT was excised from the Coomassie blue-stained gel and analyzed by mass spectrometry. MALDI-TOF-MS analysis spectrum from trypsin digestion fragments of protein band* and database analysis suggested the protein band* was PDZK1. (C) PDZK1 was identified as PTEN-CT associated protein by Western blotting. Equal amounts of GST or purified GST fusion protein beads of PTEN-CT were used respectively for pull down COS7 lysates, and the pull-down complex was subjected to Western blotting with an anti-PDZK1 antibody. (D) PTEN and PDZK1 interact in stomach tissues. Solubilized lysates from homogenized rabbit stomach tissues were subjected to immunoprecipitation with or without the anti-PTEN antibody. Co-immunoprecipitated PTEN was then probed with anti-PDZK1 antibody by Western blotting. Immunoprecipitation of the lysates with IgG antibody was used as the negative control. Representative images of Western blot analysis from three independent experiments were shown.

Fig. 2. PDZK1 interacted with PTEN via PTEN-CT and PDZ domain of PDZK1. (A) Schematic diagrams of the full length and recombinant fragments of PDZK1 and PTEN protein. (B) GST-PTEN-CT specifically binds to the PDZ domain of PDZK1. GST-PTEN-CT beads were used to pull down His-PDZK1 PDZ1, PDZ2, PDZ3 or PDZ4 fusion proteins, respectively. Precipitates were subjected to Western blotting with an anti-His antibody. A robust signal corresponding to the PDZ2 and PDZ3 domains of PDZK1 was detected in the GST-PTEN-CT pull-down complexes. (C) The overlay assay results indicate a relatively high affinity between PTEN-CT and PDZK1 PDZ domains. Nitrocellulose strips containing 1 μg of His/S-tagged PDZK1 PDZ2 or PDZ3 were incubated with GST-PTEN-CT at different concentrations ranging from 0 to 400 nM. Specific binding of PDZK1 PDZ domain did not increase significantly between 100 and 400 nM; therefore, the binding observed at 400 nM was defined as the ‘maximal’ binding. The binding observed at the other concentrations was expressed as a percentage of the maximal binding within each experiment. (D) PTEN-CT selectively associates with PDZK1. Purified GST fusion proteins corresponding to either wild-type PTEN-CT-ITKV (denoted by its last four amino acids) or pointmutated versions of the wild type (denoted by sequential replacement of each of the last four amino acids with alanine: ITKA, ITAV, IAKV, ATKV) were used to pull down the cell lysates of COS7 which expressed Myc-PDZK1. Pull down precipitates were subjected to Western blot with anti-myc antibody. Coomassie blue staining confirmed that equivalent amounts of various GST fusion proteins were present in each sample. (E) PTEN-CT selectively associates with the PDZ domain of PDZK1. Purified GST fusion proteins corresponding to either wild-type PTEN-CT-ITKV or point-mutation (ITKA) were used to pull down His-PDZ2 or His-PDZ3 of PDZK1 fusion proteins, respectively. Pull down precipitates were subjected to Western blot with anti-His antibody. Coomassie blue staining confirmed that equivalent amounts of GST fusion proteins were present in each sample. (F) PTEN selectively interacts with PDZK1 in cells. Equal amount constructs of Flag-tagged full length of wild type PTEN (Flag-PTEN-ITKV) or its V403A mutant (Flag-PTEN-ITKA) in the presence of myc-PDZK1 constructs were transiently transfected into COS7 cells respectively. After 48 h transfection, the cells were lysated and incubated with anti-Flag antibody, the immunoprecipitated complex was then subjected to Western blotting with anti-myc antibody. The data shown in all panels of these figures are representative of three to five independent experiments.

Fig. 3. PDZK1 suppressed PTEN phosphorylation and PI3K/AKT signaling activation in gastric cancer cells. (A–B) PDZK1 inhibited PTEN phosphorylation. Lysates of BGC-PDZK1 (A), BGC-PDZK1 cells which were transiently transfected with siPDZK1#1 and siPDZK1#2 (B), or their

control cells were collected and subjected to Western blot analysis by using specific antibodies which recognized phospho-PTEN (S380/T382/T383) or total PTEN. (C) Inhibition of PTEN phosphorylation by PDZK1 required PDZK1/PTEN interaction. COS7 cells were co-transfected with myc-PDZK1, and Flag-PTEN-ITKV or Flag-PTEN-ITKA. After 48 h transfection, lysates were analyzed by using anti-PTEN or anti-phospho-PTEN (S380/T382/T383) antibodies in Western blotting. (D) PDZK1 inhibited AKT activation in a dose dependent manner. BGC-823 cells were transiently transfected with increased concentration of myc-PDZK1 constructs. Then lysates were collected and subjected to Western blot analysis by using indicated antibodies. (E) Knock down of PDZK1 enhanced the phosphorylation of AKT. BGC-PDZK1 cells were transiently transfected with siPDZK1#1 and siPDZK1#2 to knock down PDZK1 expression and cell lysates were analyzed by Western blot analysis using indicated antibodies. (F) PDZK1 inhibition of AKT activation was PTEN-dependent. BGC-823 cells which stably expressed shPTEN were transiently transfected with myc-PDZK1 in the absence or presence of serum (10%, 15 min) stimulation. The lysates were collected and subjected to Western blot analysis by using specific antibodies as indicated in the figure. Western blot analysis was repeated at least three times and representative images of were shown.

Fig. 4. PDZK1 inhibited proliferation of gastric cancer cells. (A, B) PDZK1 inhibited gastric cancer cell proliferation. BGC-823 cells which stably overexpressed PDZK1 (BGC-PDZK1) and the vector (BGC-Control) (A) or AGS gastric cancer cells which stably overexpressed PDZK1 (AGS-PDZK1) and the control vector (AGSControl) (B) were seeded in 96-well plates. Proliferative cells were determined at different time as indicated in the figure. (C, D) Knock down of PDZK1 expression promoted proliferation of gastric cancer cells. BGC-823 cells (C) or AGS cells (D) were transfected with PDZK1 shRNA#1, PDZK1 shRNA#2 or its scrambled control. Cell proliferation was determined by CCK-8 assay. (E) PDZK1 overexpression inhibited the colonyforming ability of gastric cancer cells. The number of colonies was counted in BGC-PDZK1 and AGSPDZK1 cells and their control groups. Histograms represent the quantification of the number of colonies. (F) Knockdown of PDZK1 expression suppressed the colony-forming ability of gastric cancer cells. The colony formation assays were performed as described in the “Methods” and representative images are shown. The number of colonies was counted for BGC823-shPDZK1 and AGS-shPDZK1 cells and their control groups. (G) PDZK1 inhibition of BGC-823 cell proliferation was PTEN-dependent. BGC-823 cells in which endogenous PTEN was stably knocked down by shRNA (shPTEN) or the scramble (shControl) were transiently expressed PDZK1 or control vector and then planted in 96-well plate. Cell proliferation was determined by CCK-8 assay. All data are represented as the mean ± SD for three independent experiments (* represents $P < 0.05$, NS represents not significant).

Fig. 5. PDZK1 inhibited BGC-823 gastric cancer cell proliferation and PTEN phosphorylation in xenograft tumors. (A) Representative image and tumor weight of BGC-823 xenografts. Upper panel: The photograph of dissected tumors from the mice xenografted with BGC-PDZK1 or control cells. Lower panel: Tumor weights of xenograft tumors in BGC-PDZK1 group were significantly smaller than those in the BGC-823 control group. BGC-823-control or BGC-PDZK1 cells were subcutaneously injected into the nude mice. The mice were sacrified at 7 weeks after tumor cell implantation. (B) The growth curve of subcutaneous xenograft tumor from BGC-823 cells in nude mice. One week after implantation, tumor size was calculated every 7 days. Values were expressed as the mean ± SD of 10 mice in each group (Repeated-measures analysis of variance, $P < 0.05$). (C) Phospho-PTEN (S380/T382/T383) expressed at lower levels in BGC-PDZK1 xenograft tumors. Representative images for PDZK1, PTEN and phospho-PTEN (S380/T382/T383) expression from Western blot of homogenates of tumor xenografts were shown. Independent experiments were repeated for three times. (D) IHC analysis of xenograft tumors showed a relatively weak staining of phospho-PTEN (S380/T382/ T383), phophos-AKT and Ki67, but a higher number of TUNEL staining positive cells in BGC-PDZK1 xenograft tumors. Representative images for PTEN, phosphoPTEN (S380/T382/T383), AKT, phospho-AKT, Ki67 and TUNEL staining from immunohistochemistry analysis of tumor xenografts were shown. Independent experiments were repeated for three times. Scale bars: 20 μm (×400 magnification).

Fig. 6. Low levels of PDZK1 were correlated with poor tumor progression. (A) PDZK1 protein was significantly low expressed in GC patients (n = 158). Values were expressed as median ± interquartile range, P < 0.0001, nonparametric Mann–Whitney test. (B) Representative images of PDZK1 IHC staining in GC patients (Right) and its adjacent normal tissue (Left). Scale bar, 50 μm. H&E, hematoxylin and eosin. (C) Low levels of PDZK1 mRNA were detected in TCGA dataset of GC patients (n = 450). Values were expressed as mean ± SD (P < 0.05, Unpaired t-test). (D) Downregulation of PDZK1 in clinical GC tissues is correlated with advanced clinical stage. The mRNA levels of PDZK1 were analyzed in GC patients obtained from TCGA database. The PDZK1 expression was correlated with higher T stage in GC patients in TCGA dataset.(E–F) Expression levels of PDZK1 at different clinical grades of GC. The tumor tissues collected from Chinese GC patients (E) or TCGA (F) were further divided into high, moderate and low grade according to the pathological staining. PDZK1 levels were reduced with the increased tumor grade of GC. (G–H) Kaplan-Meier survival analysis of patients was performed according to PDZK1 expression levels in tumor samples from two independent cohorts of Chinese GC patients (G) and GC patients from TCGA dataset (H). Patients were divided into high (greater/equal to average) and low (less than average) PDZK1 expression groups. The overall survival (OS) rates were significantly higher in the group with high PDZK1 levels (P < 0.05, log-rank test). Scale bars: 20 μm (×400 magnification).

Fig. 7. PDZK1 expression was negatively associated with the activation of PTEN-mediated PI3K/AKT signaling in GC patients. (A) Negative correlation of PDZK1 expression and PTEN phosphorylation was in GC and adjacent normal tissue from Chinese gastric cancer cohort. Left, GC and adjacent tissue specimens were analyzed by IHC staining with the indicated antibodies (n = 10). Scale bars: 100 μm (×100 magnification, top panel); Scale bars: 20 μm (×400 magnification, bottom panel). Right, the signal intensity of PDZK1, PTEN and phospho-PTEN (S380/T382/T383) was quantified by IPP software. Results represent the mean ± SD of 10 samples. * P < 0.05 with respect to adjacent tissue. (B) PTEN signaling was inactivated in poor prognosis of GC patients. Prognosis data of 274 gastric cancer patients were obtained from TCGA database. Enrichment plots of GSEA for gene signatures of PTEN inactivation significantly enriched in groups with poor prognosis (< 5 years survival) as compared with good prognosis groups (≥5 years survival) (FDR = 0.001). (C) PTEN signaling was inactivated in GC groups with low PDZK1 levels. GC patients from TCGA GC dataset were divided into high (higher/equal to median, n = 207) and low (less than median, n = 208) PDZK1 mRNA expression groups. Enrichment plots of GSEA for the up-regulated genes upon PTEN inhibition significantly enriched in groups with low PDZK1 levels (FDR = 0.06). (D) Activation of AKT signaling genes was enriched in TCGA GC groups with low PDZK1 levels. Enrichment plots of gene expression signatures for AKT signaling activation were enriched in TCGA GC groups with low PDZK1 levels by GSEA. (E) Cell proliferation was activated in GC groups with low PDZK1 levels. Enrichment plots of gene expression signatures for proliferation enriched in GC groups with low PDZK1 levels by GSEA in the same TCGA dataset as panel C. (F) Cell apoptosis was activated in GC groups with high PDZK1 levels. Enrichment plots of gene expression signatures for apoptosis enriched in TCGA GC groups with high PDZK1 levels by GSEA.